S. Crauwels, J. Steensels, G. Aerts, K. A. Willems, K. J. Verstrepen and B. Lievens

Brettanomyces Bruxellensis, Essential Contributor in Spontaneous Beer Fermentations Providing Novel Opportunities for the Brewing Industry

Recently, the non-conventional, wild yeast *Brettanomyces*, with *B. bruxellensis* (teleomorph *Dekkera bruxellensis*) as the most commonly encountered species, has gained more and more attention in academic research as well as the food and beverage industry. *Brettanomyces* is a distant relative of the classic brewing yeast *Saccharomyces* cerevisiae and is especially known for its ambiguous role in food and beverage fermentations. Whilst still mainly considered a spoilage organism responsible for off-flavor production in wine, cider and dairy products, *Brettanomyces* yeasts can also add desirable flavors to fermented beverages such as lambic and gueuze beers, accounting for many of the typical organoleptic characteristics of the beer. Today, the unique aromatic properties of *Brettanomyces* and its opportunities for beer brewing are increasingly recognized, with more and more (artisan) brewers adding it deliberately to their fermentations. In this review, we give a comprehensive overview of the currently available information on *Brettanomyces* yeasts with relevance for the brewing sector, emphasizing *B. bruxellensis.* First, the history and taxonomy of *Brettanomyces* is discussed. Secondly, we discuss the dual role of the yeast in fermented beverages by contrasting its role in beer and wine: in certain beer styles it plays a crucial role, in wine it is considered one of the most important spoilage microbes. In this regard we also discuss some of its most important phenotypic characteristics for the food and beverage industry, including flavor and off-flavor production, and focus on its capability to thrive in industrial fermentations. Finally, we review the most important detection and identification methods and address some opportunities for the brewing industry exploiting *Brettanomyces* yeasts.

Descriptors: (Off-)flavors, lambic beer, sour beers, spontaneous fermentation, volatile phenols

1 Introduction

For millennia, humans have taken advantage of fermentation processes to improve the shelf life and safety of diverse foods and beverages. Additionally, fermentation can add a variety of desirable flavors to the finished products or eliminate unpleasant flavors [1]. Whilst most fermentation processes were initially conducted spontaneously by microorganisms from the surrounding environment, nowadays they are performed by single strain starter cultures to produce a product of consistent quality [2]. In alcoholic fermentations, these starter cultures generally consist of a single strain of *Saccharomyces cerevisiae* or a closely related species such as *S. pastorianus* [3]. However, in certain conditions or for certain fermentation processes, the physiological boundaries of *Saccharomyces* strains limit their applicability. Additionally, a lot of the complexity or subtle aromatic notes that are inherent to spontaneous fermentations may get lost in pure culture fermentations. Moreover, selecting (or developing [4]) a single strain with all characteristics necessary for an efficient and high-quality fermentation proves a real challenge for the food and beverage industry [5]. Therefore, there is currently a strong interest in other yeasts than *Saccharomyces* (so-called 'non-conventional yeasts') that are able to complement or replace traditional *Saccharomyces* brewing strains [6]. This interest mainly lies in the ability of several non-conventional yeast species to produce low-alcohol beers and/or to produce beers with a peculiar aroma profile [7–9]. Further, there is a growing interest in sour beers that are the result of spontaneous fermentation relying on natural inoculum, especially in the USA where hundreds of commercial examples of American sour ales have been released [5, 10]. In this regard, *Brettanomyces* yeasts, with *B. bruxellensis* (teleomorph *Dekkera bruxellensis*) as the most commonly encountered species, have gained more and more attention in the brewing industry over the last few years. *B. bruxellensis* plays a key role in spontaneous beer fermentation processes, such as the production of the lambic and gueuze (i.e. a blend of old and new lambic) beers typically produced in or near

Authors

Dr. Sam Crauwels[1,2], Dr. Jan Steensels[2,4,5], Prof. Guido Aerts[2,3], Prof. Kris A. Willems[1,2], Prof. Kevin J. Verstrepen[2,4,5] and Prof. Bart Lievens[1,2];
[1] Laboratory for Process Microbial Ecology and Bioinspirational Management (PME&BIM), Department of Microbial and Molecular Systems (M2S), KU Leuven, Sint-Katelijne Waver, Belgium;
[2] Leuven Institute for Beer Research, KU Leuven, Leuven, Belgium;
[3] Laboratory of Enzyme and Brewing Technology (EFBT), M2S, KU Leuven, Ghent, Belgium;
[4] Laboratory for Genetics and Genomics, M2S, KU Leuven, Leuven, Belgium;
[5] Laboratory for Systems Biology, VIB, Leuven, Belgium;
*corresponding author: bart.lievens@kuleuven.be

the Senne river valley, an area near Brussels, Belgium [11–13], Berliner Weiße (Berlin wheat beer style) [14], and American coolship ales, mimicking the lambic beer production method [13]. In these fermentations, *Brettanomyces* lives in perfect harmony with various other microbial groups, such as acetic acid bacteria and lactic acid bacteria, and accounts for many of the typical organoleptic characteristics of the beer. German Berliner Weiße is a low-gravity wheat beer fermented with *S. cerevisiae* and *Lactobacillus spp.* in mixed culture. The conventional wisdom has long said that wild yeasts did not belong in Berliner Weiße. However, more recently it has been found that the traditional aroma of Berliner Weiße is actually due to secondary fermentation by *Brettanomyces* [14]. The unique aromatic properties of *B. bruxellensis* are increasingly recognized, with more and more artisanal brewers adding it to their fermentations, either as a pure culture or in combination with more traditional brewing strains. Besides, its unique flavor profile makes *Brettanomyces* yeasts highly attractive for the production of novel specialty alcoholic beverages [15]. However, the role of *Brettanomyces* in the food and beverage industry is confounded and double. While they are crucial contributors to the flavor profile of certain specific specialty beers, they are also reported as main spoilage microbes in diverse foods and beverages, mostly due to their typical aroma profile, which can be described as 'burnt plastic', 'barnyard', 'medicinal', 'horse sweat', and 'leather' amongst some other unpleasant flavors [16, 17]. Moreover, spoilage of wine by *B. bruxellensis*, for example, is considered the most important microbiological issue in the wine industry, by which, unfortunately, the beneficial effects of *Brettanomyces* have been generally overshadowed in the food and beverage industry.

Table 1 Overview of old and new taxonomical classifications of *Brettanomyces* and *Dekkera species**

Old classification	Substrate of isolation	New classification
B. sphaericus	Cucumber brine	*C. etchellsii*
B. petrophilum	NA	*C. parapsilosis*
B. italicus (var. membranifaciens)	Wine	*C. stellata*
B. versatilis	Cucumber brine	*C. versatilis*
D. custersiana	Beer	*B. custersianus*
B. custersianus	Beer, olives, carbonated beverages, wine	
D. naardenensis	Carbonated beverages	*B. naardenensis*
B. naardenensis	Carbonated beverages, beer	
B. nanus	Beer	*B. nanus*
D. nana	Beer	
Eeniella nana	Beer	
B. nonanus	Beer	
B. anomalus	Beer, cider, sherry wine, tequila	*B. anomalus/ D. anomala*
B. cidri	Cider	
B. dublin(i)ensis	Beer	
Candida beijingensis	NA	
Torulopsis cylindrical	Beer	
Monilia vini	Wine	
Mycotorula claussenii	NA	
Oospora vini	Wine	
D. anomala	Carbonated beverages, kefir, beer, sherry wine, cider	
B./D. abstinens	Carbonated beverages	*B./D. bruxellensis*
B. bruxellensis var. vini/bruxellensis/lentus/non-membranifaciens	Beer	
B. custersii	Beer, wine, sourdough	
B. intermedius	Carbonated beverage, beer, wine	
B. lambicus	Beer	
B. patavinus	Wine	
B. schanderii	Beer	
B. vini	Wine	
D. intermedia	Tea beer	
D. lambica	Beer	
Mycotorula intermedia	Wine	
B./D. bruxellensis	Kefir, sherry wine, kombucha, cider, bioethanol, sourdough, yoghurt, black olives, carbonated beverage	

*Original sources of isolation are indicated for each species. B =*Brettanomyces*, D = *Dekkera*, C = *Candida*, NA = not available. For original references we refer to *Steensels* et al. (2015) [20]

In this review, we give a concise overview of the currently available information on *Brettanomyces* yeasts with relevance for the brewing sector, emphasizing *B. bruxellensis*. First, we address the history and taxonomy of the *Brettanomyces* genus. Secondly, we discuss the ambiguous role of the yeast in fermented beverages by contrasting its role in beer and wine. In this regard we also discuss some of its most important phenotypic characteristics relevant for the food and beverage industry, including flavor and off-flavor production, and elaborate on its important capability to thrive in industrial fermentations. Lastly, we review the most important detection and identification methods and address some opportunities for the brewing industry exploiting *Brettanomyces* yeasts. More fundamental and detailed studies on this yeast are given elsewhere in recent review papers [18–20].

2 History and taxonomy of *Brettanomyces*

Brettanomyces is an anamorphic yeast genus in the family *Saccharomycetaceae* (*phylum Ascomycota*). The first reference to the genus goes back to 1904, when Niels Hjelte Claussen isolated his so called 'British fungus' (Greek: 'brettano', British; 'myces', fungus) at the Carlsberg brewery, where it

was held responsible for performing a secondary fermentation and development of characteristic flavors in British beers [21]. Following this discovery, it was not until the 1920s, when more isolates were obtained from lambic beers, that *Brettanomyces* was proposed as a genus [22]. The species name '*bruxellensis*' (Latin: from Brussels) was proposed to tribute to the role of this species in the production of Belgian lambic and gueuze beers, which are traditionally brewed in the area of Brussels (Belgium). In the following years, *B. bruxellensis* has been isolated from several industrial fermentations and fermented products such as wine [23, 24], cider [25, 26], kombucha tea [27], kefir [28], and olives [25]. Further, the species is frequently isolated as a contaminant in bioethanol production sites [29–31]. The only source from which *B. bruxellensis* has been isolated that is not associated with industrial settings is grape berries [32], illustrating its close association with man-made ecological niches.

Over the years, many different *Brettanomyces* species have been suggested and the names of these species were freely used in scientific publications. Moreover, there have been many reclassifications over the years, making direct comparisons between old and more recent research papers often challenging. In table 1 an overview of many initial and current species names are given, illustrating the complexity of *Brettanomyces* taxonomy. A first attempt to describe the genus *Brettanomyces* comprehensively was performed by *Custers* in 1940 based on a number of phenotypic features [23]. In 1960, the formation of ascospores was observed in some strains and the genus *Dekkera* (a name chosen in honor of Nellie Margaretha Stelling-Dekker, a pioneer of yeast systematics) was introduced in the taxonomy as the teleomorphic (sexual) counterpart of *Brettanomyces* [33]. In the first edition of their manual on yeast characteristics and identification, Barnett and co-workers [34] described the following nine *Brettanomyces* and *Dekkera* species: *Brettanomyces* abstinens, *B. anomalus, B. claussenii, B. custersianus, B. custersii, B. lambicus, B. naardenensis, Dekkera bruxellensis* and *D. intermedia*. Nowadays, based on molecular data, it is agreed that the genus encompasses five species, including the anamorphs *B. anomalus, B. bruxellensis, B. custersianus, B. naardenensis*, and *B. nanus*, with teleomorphs existing for the first two species, *D. anomala* and *D. bruxellensis* [35]. Recent reconstruction of phylogenies based on large numbers of orthologous genes position *Brettanomyces* in a group with, among some others, *Ogataea polymorpha*, that appears to have diverged from the progenitor of the so-called 'CTG-clade' (containing e.g. *Candida albicans, Debaryomyces hansenii* and *Sheffersomyces stipitis*) [36], after sharing a common ancestor with *S. cerevisiae* [18, 37]. The lineages of *B. bruxellensis* and *S. cerevisiae* separated approximately 200 million years ago [38], around the same time the first mammals emerged on earth. After the first description of spore formation [39], spores have not been reported again [19]. Therefore, and also because the name '*Brettanomyces*' is used more commonly in the food and beverage industry, we will use the name '*Brettanomyces*' over '*Dekkera*' in this manuscript.

3 *Brettanomyces*: crucial in (some) beer, unwanted in (most) wine

B. bruxellensis plays an essential role in beer fermentation processes relying on a natural inoculum such as the lambic and gueuze. Other examples of beer styles involving *Brettanomyces* yeasts include acidic ales produced in the North-West of Flanders (Belgium), American coolship ales inspired by lambic beers, Berlin style wheat beers, and certain Belgian Trappist beers (and other ales) with in-bottle refermentation by *Brettanomyces* [2, **Fehler! Verweisquelle konnte nicht gefunden werden.**, 14, 40]. Lambic beers are among the oldest types of beers still brewed, and are traditionally brewed during the colder months of the year (October to March). Cold nights are needed to lower the temperature of cooked wort over-night to about 20 °C in the so-called 'coolship'. Subsequently, the cooled wort is assumed to be inoculated with specific microbes from the air or the brewery environment, and is transferred into wooden casks which are stored at cellar or ambient temperatures, i.e. typically between 10 and 25 °C. Subsequently, the wort ferments and the lambic beer matures in the same casks. The end product is a non-carbonated sour lambic beer that mainly serves as a base for gueuze or fruit lambic beers that are refermented in the bottle [41].

In general, the lambic beer fermentation process consists of four phases, each characterized by a typical microbial community. The initial phase starts after 3 to 7 days of fermentation and proceeds until 30 to 40 days. This phase is characterized by a broad microbial diversity mainly consisting of *Enterobacteriaceae* [42] along with yeasts such as *Kluyveromyces sp., Naumovia dairensis, Pichia sp., Rhodotorula sp.* and *Saccharomyces uvarum*, causing a drop in pH (down to pH 4.6) and a slight increase in ethanol concentration [41, 43]. Subsequently, the main fermentation is characterized by the presence of *Saccharomyces* species such as *S. cerevisiae, S. bayanus* and *S. uvarum*, and leads to an increase of ethanol concentration [41, 43]. After three to four months of fermentation, the acidification phase occurs which is characterized by a high density of lactic acid bacteria, i.e. *Pediococcus spp.*, and (in lower numbers) acetic acid bacteria, providing sourness to the beer. After four to eight months of fermentation most mono-, di-, and trisaccharides (such as glucose, fructose, sucrose, maltose and malto-triose) are depleted and ethanol concentration has increased to about 5–6% v/v. This highly specific environment causes a shift in the yeast community from *Saccharomyces* to *Brettanomyces spp.* (mostly *B. bruxellensis*) [41, 43]. *Brettanomyces* combines high ethanol tolerance and the ability to super attenuate (or 'overferment') the wort, i.e. utilize complex carbohydrates such as maltotetraose and maltopentaose, and can reach cell counts of 10^4–10^5 cells per ml, allowing them to establish the typical 'Brett' flavors (see below) [2, 44]. The final maturation phase, during which the wort is gradually attenuated, starts after ten months of fermentation and is characterized by a decrease of lactic acid bacteria [41, 43].

Whereas their presence in these specialty beers is imperative, *Brettanomyces* yeasts are also considered to be some of the worst spoilage microbes in wine, causing substantial economic losses [45]. As with beer, the flavors of wine are the products of complex interactions between many microorganisms, including *S. cerevisiae* and some others. *S. cerevisiae* is the primary yeast used in wine-making, but other fungi, yeasts and bacteria may also contribute to the fermentation. Many of these species occur naturally on the grapes and flourish in the initial stages of the fermentation before being killed by the rising ethanol concentration [46]. Other species originate from the winery environment itself,

surviving on the walls of the winery, on the interior surfaces of presses and fermentation tanks, or in the wood of the (oak) barrels [47], enabling them to colonize the fermenting grape must or the maturing wine. *B. bruxellensis* is such species, which can survive for extended periods in the winery and negatively influence the wine quality. More specifically, *B. bruxellensis* may perform a secondary fermentation after *S. cerevisiae* has completed alcoholic fermentation, altering the flavor profile. Infected beverages develop unpleasant aromas, also referred to as 'Brett' taints (see below) [16, 17]. However, at low levels, some winemakers agree that the presence of these compounds can have a positive effect on wine, contributing to complexity, and giving an aged character to some young red wines. Some wines even rely on *Brettanomyces* to give their distinctive characteristic aroma profile, such as the French Château de Beaucastel wines. However, when the levels of these volatile compounds greatly exceed the sensory threshold, the perception is almost always negative.

4 'Brett character': flavors and off-flavors associated with *Brettanomyces*

Brettanomyces can strongly affect the aroma of fermentation products. Many different terms, including, amongst some others, 'barnyard', 'clove', 'horsy', 'leathery', 'medical', 'mousy', 'smoky', and 'spicy', but also 'floral', 'tropical', and 'fruity' have been used to describe the aroma profile of *Brettanomyces*, colloquially referred to as 'Brett flavor' or 'Brett character'. Below the most important and industrially relevant flavors associated with *Brettanomyces* are discussed.

4.1 'Mousy' taints

'Mousy' taints are often encountered in wines infected with *Brettanomyces* or lactic acid bacteria. The aromas associated with *Brettanomyces* 'mousiness' are the result of pyridines synthesized from lysine and ethanol, such as 2-acetyl-3,4,5,6-tetrahydropyridine, 2-acetyl-1,2,5,6-tetrahydropyridine and 2-ethyl-3,4,5,6-tetrahydropyridine [48], although the absolute concentrations may vary between different species and strains [49]. The aromas imparted are characterized as 'caged mice' and are sometimes similar to 'cracker biscuits', but under low pH conditions they can be perceived as 'metallic' or 'bitter' [49]. The aromas are usually only perceived after swallowing and the flavor can persist for more than 10 minutes [48]. Notwithstanding the huge impact of these compounds on the quality of beverages, surprisingly to date still little is known about these compounds and their production by *Brettanomyces spp.*

4.2 Volatile phenolic compounds

Volatile phenolic compounds are the key molecules responsible for some of the most recognized aromatic characteristics associated with *Brettanomyces* species, and result in the typical 'Brett flavors' described as 'barnyard', 'clove', 'horsy', 'leathery', 'medicinal', 'smoky' and 'spicy'. Four compounds have been commonly attributed to the phenolic flavor, including 4-vinylguaiacol (4-VG), 4-vinylphenol (4-VP), 4-ethylguaiacol (4-EG) and 4-ethylphenol (4-EP) [16, 49, 50]. Nevertheless, it was recently discovered that 4-vinylcatechol (4-VC) and 4-ethylcatechol (4-EC) may also have a role in the aroma of fermented beverages, especially in wine and cider [51, 52]. The vinyl derivatives, which are the precursors for the ethyl compounds, have a similar taste as the ethyl derivatives but have lower flavor thresholds. These phenolic volatiles are produced from non-volatile organic acids such as hydroxycinnamic acids that are, for example, naturally present in grape must, wine, or malted barley. Therefore, their production depends on the fermentation medium, since precursor composition and concentration may vary significantly. For example, *Brettanomyces* contamination occurs much more frequently in red wines where extraction of precursors of volatile phenols is more intense than for white wines [17, 53]. Additionally, apart from the fermentation medium, production of these volatile phenols was shown to vary between different *B. bruxellensis* strains [54]. Interestingly, there seems to be a correlation between strain origin and volatile phenol production, as only wine strains produced detectable amounts of 4-EG and 4-EP when inoculated in red wine (*Crauwels* et al., manuscript in preparation),

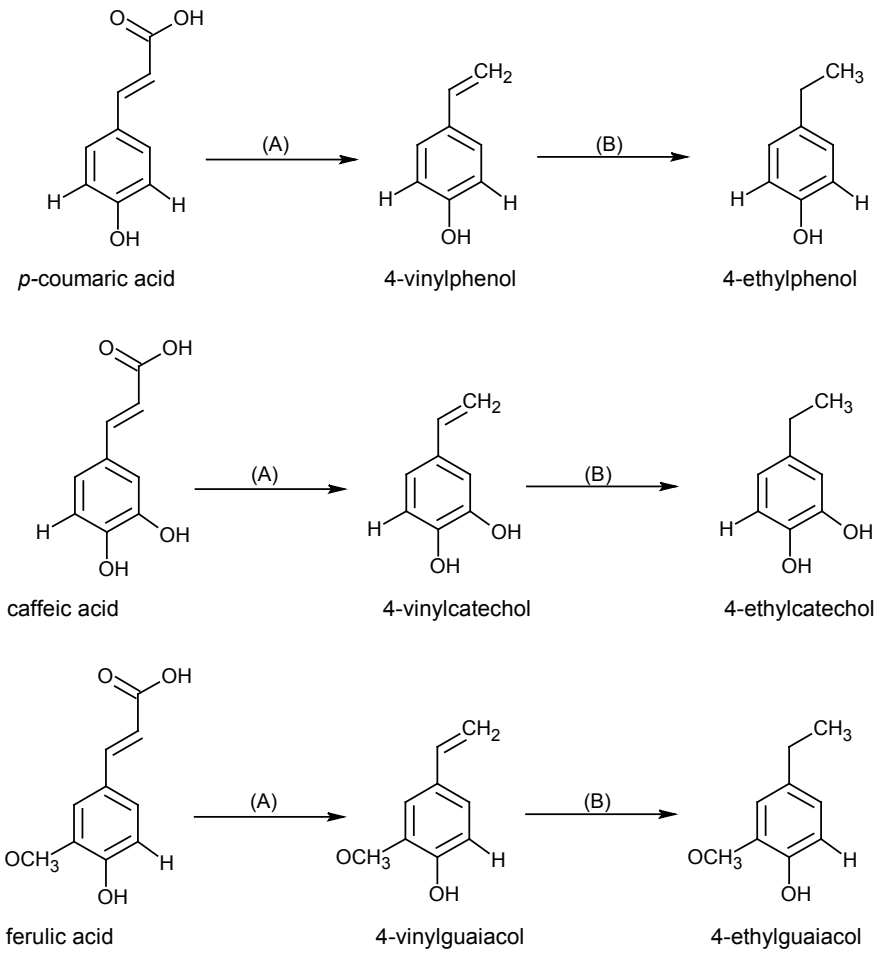

Fig. 1. Conversion of hydroxycinnamic acids (e.g. present in grape must or malt) in a 2-step enzymatic process: a hydroxycinnamate decarboxylase (A) followed by a vinylphenol reductase (B) resulting in the 'Brett' associated aromas 4-vinylphenol, 4-vinylcatechol, 4-vinylguaiacol and 4-ethylphenol, 4-ethylcatechol and 4-ethylguaiacol. *Brettanomyces* is almost unique among other yeasts because of its ability to form the ethyl derivatives. *Saccharomyces*, for example, can only produce the vinyl derivatives

suggesting differences in the physiological behavior between strains from a different ecological origin. Notably, *Brettanomyces* is one of the few yeasts able to convert hydroxycinnamic acids into the strong smelling ethyl derivatives, while many other organisms, such as *S. cerevisiae*, only form vinyl derivatives with no further conversion to ethyl derivatives, explaining why these phenolic flavors are typically associated with *Brettanomyces* [16, 55]. For example, the hydroxycinnamic acids ferulic acid and p-coumaric acid are rapidly converted by *Brettanomyces* to 4-EG and 4-EP due to sequential activity of two enzymes, a hydroxycinnamate decarboxylase and a vinyl phenol reductase, the latter which is specific for *Brettanomyces* (Fig. 1) [56–57]. Surprisingly, to date still little is known about the genes encoding the enzymes involved in the production of these phenolic metabolites. Only recently, *Godoy* and coworkers [58] described a gene encoding a phenolic acid decarboxylase in *B. bruxellensis* (DbPAD gene), whose function was verified by heterologous expression in *S. cerevisiae*. Additionally, de *Souza Liberal* et al., 2012 [59] reported the existence of two genes in *B. bruxellensis* that encode for paralogues of the enzyme phenylpyruvate decarboxylase (DbARO10). Expression of these paralogous genes of DbARO10 showed that both respond to the presence of p-coumaric acid, indicating that there may be alternative or additional decarboxylases in *B. bruxellensis*. With regard to the vinyl phenol reductase enzyme, only recently the putative enzyme was purified and sequenced. Further, it was found to possess both vinyl phenol reductase and superoxide dismutase activities and was univocally identified as a superoxide dismutase in the *B. bruxellensis* AWRI 1499 genome [60].

Interestingly, whilst 4-EG and 4-EP strongly contribute to off-flavors in wine, the same compounds are considered desirable in lambic and various acidic ale beers. The difference between the perceived effect of *Brettanomyces* on wines and beers may be explained by the difference in relative concentration of these volatile phenols. In beer, the concentration of 4-EG (clove-like or spicy aroma) is higher than that of 4-EP (medicinal, barnyard aroma); in wine, this is the opposite [49]. Wine spoilage by *Brettanomyces* is generally characterized by a ratio of 4-EG and 4-EP of less than one. In contrast, in beer the ratio is generally twenty times larger than one [61,62]. However, the ratio of 4-EG and 4-EP also varies substantially between wines, ranging from 1:3 to 1:40 [63]. The reason for these differences in wine as well as between wine and beer are not yet fully understood, but it is likely to assume that they are caused by the combined effect of differing ratios between coumaric and ferulic acids and of different strains with some strains being more effective in producing one compound over the other [63, 64; Crauwels et al., manuscript in preparation]. Apart from heavily influencing the aroma profile of various food products, production of ethyl phenols might also pose a clever strategy of *Brettanomyces* to travel to new environments, since it was recently shown that these compounds can serve as an attractant for fruit flies [65] and can therefore play a crucial role in the dispersal of the yeast through insect vectors, a mechanism which has also been described for *S. cerevisiae* [66].

4.3 Volatile fatty acids

Brettanomyces has been shown to produce large amounts of volatile fatty acids in anaerobic fermentation conditions such as isovaleric acid. Many of these acids can have an unpleasant rancid odor and/or taste, which may be noticeable in spoiled wine, or young *Brettanomyces* beers before these acids are esterified [67]. In fact, together with the mousy off-flavors and phenolic compounds mentioned above, isovaleric acid is the main contributor to the undesirable *Brettanomyces* character in wine [17]. Additionally, it is believed to affect the overall perception or intensity of volatile phenolic compounds in fermented products [49]. In lambic isovaleric acid gives the beer its sweaty and cheesy flavors and odors. The concentration of isovaleric acid in lambic beers commonly ranges between 2 and 3 ppm, although also some commercial lambic beers were found in which no isovaleric acid could be detected [68].

4.4 Volatile esters

Volatile esters are an important group of aromatic compounds as they are responsible for the fruity or flowery character in fermented beverages [69]. *Brettanomyces* is capable of forming high concentrations of several ethyl esters (such as ethyl acetate, ethyl lactate, ethyl caprate and ethyl caprylate), while it can actively break down certain acetate esters (such as isoamyl acetate) by its esterase activity [67, 70]. In later stages of lambic beers fermentations, which are typified by a complex microbial cocktail of *Brettanomyces* yeasts and various bacterial species, the ester fraction typically constitutes of a very low amount of isoamyl acetate and significant amounts of ethyl acetate, ethyl caprate, ethyl caprylate and ethyl lactate [12]. While the average concentration of ethyl acetate is between 8 and 48 ppm for traditional beers, it is between 33.4 and 67.6 ppm for filtered gueuze and between 60.9 and 167 ppm in unfiltered gueuze [71]. The concentration of ethyl lactate in lambic beers has been determined to be above 400 ppm, which is well above the taste threshold of 50 ppm and the odor threshold of 14 ppm [72]. Ethyl caprylate and ethyl caprate, which are normally absent in lagers or only present in small concentrations in ales, are considered to be typical aroma and flavor compounds of lambic and gueuze beer, giving these beers their wine and fruity flavor. The concentration of ethyl caprylate may go up to 5.7 ppm in certain gueuze beers [68].

4.5 Acetic acid

In the presence of oxygen, *Brettanomyces* strains are capable of producing acetic acid. Depending on the brewer's palate and the degree of acetic production, this can be a desirable or undesirable trait. Acetic acid is generally considered negative in fermented beverages when concentrations of 1.2–1.3 g/L are reached. In lambic beers, acetic acid concentrations typically vary from 0.4 to 1.2 g/L (in wine from 0.2 to 0.6 g/L). The wide range of acetic acid concentrations in lambic beers can potentially be explained by the presence of different acetic acid bacteria and/or different *Brettanomyces* strains. The acetic acid produced may also be used in the synthesis of acetate esters such as ethyl acetate [67, 70].

4.6 Sugar-bound flavor-active compounds

In addition to the typical *Brettanomyces* flavors mentioned above, *B. bruxellensis* may introduce additional flavors into the fermented products. Besides the presence of flavor-active volatile compounds in a free form, fruits, flowers and other plant parts that are often used in food and beverage fermentations contain volatiles that are

'locked' in glycosidically bound sugars, resulting in water soluble, odorless compounds. These 'locked' flavors can be released by β-glucosidase enzymes, which are for example found in *Brettanomyces* strains but not in *S. cerevisiae* [73]. This makes *Brettanomyces* very well suited for the production of novel alcoholic beverages that are enriched with natural flavors and aromas from hops, fruits and other plant parts that *Saccharomyces* yeasts typically cannot produce [73–75]. Moreover, recent purification and characterization of the *B. anomalus* β-glucosidase enzyme revealed that it can act at a higher pH (5.75) and lower temperature (37 °C) than currently available commercial β-glucosidases, providing new opportunities of this enzyme for bioflavoring of certain beverages (*Vervoort* et al., manuscript in preparation). Compared to currently available enzymes, the *B. anomalus* enzyme showed an increased release of particular aglycones, including eugenol and geraniol in cherry beer and linalool oxide, benzyl alcohol and methyl salicylate in forest fruit milk. Interestingly, recent studies have shown that some *B. bruxellensis* strains contain two distinct β-glucosidase genes, while other strains only contain one of the two genes [76, 77]. While this genomic deletion could be linked to the inability of utilizing some beta-linked sugars by some strains of *B. bruxellensis* [77], further research is needed to investigate the exact role of these β-glucosidases in the flavoring capability of *B. bruxellensis* strains.

5 *Brettanomyces:* a highly specialized fermentation yeast

Brettanomyces is commonly associated with man-made ecological niches, such as industrial fermentation processes (beer, wine, bioethanol, soft drinks, dairy products, sourdough,…). A common thread in these niches is the presence of harsh and limiting environmental conditions that are disastrous for many microbes: low pH, high ethanol concentrations, low oxygen concentrations, the absence of readily fermentable nitrogen and carbon sources, etc. While resistance to these stressors is not uncommon in microbes, there are only a few species that combine all of these features. One such species is *B. bruxellensis*, which has evolved as a highly specialized fermentation organism [3]. *B. bruxellensis* grows well between 19 °C and 35 °C, with optimal growth rates between 25 °C and 28 °C [78]. Nevertheless, growth, productivity and ethanol production are only slightly influenced by temperature [79, 80], making *Brettanomyces* very well suited to thrive in different fermentation processes. Further, several *Brettanomyces* strains have developed resistance against sulfite [81], which is commonly used as a disinfectant in the wine industry, where there is no boiling step to disinfect the fermentation medium like in beer brewing. Similar to *S. cerevisiae, B. bruxellensis* is Crabtree-positive, i.e. favoring fermentation over respiration in the presence of oxygen, enabling the yeast to outcompete other, ethanol-sensitive microorganisms [38, 82]. Hence, most *Brettanomyces* strains are resistant against high ethanol concentrations (up to 14.5–15 % (v/v) [83]), a trait required to survive in a fermentation environment. Interestingly, *B. bruxellensis* seems to have evolved an additional strategy to outcompete other microbes. Besides producing ethanol, they are also capable of producing, accumulating and later consuming acetic acid in aerobic conditions and withstand the resulting low pH [38]. Further, despite being a slow grower, *B. bruxellensis* is able to withstand nutrient-poor environments. For example, whereas *B. bruxellensis* preferably uses ammonium ions as nitrogen source, some strains can also use nitrate as a sole nitrogen source [76, 77, 81, 84], providing the strains a competitive advantage in nitrogen poor niches (such as soft drinks or late stages of beer and wine fermentations) over other yeasts that cannot use nitrate such as *S. cerevisiae* [85]. Using whole genome sequencing, the inability to utilize nitrate by some strains could be linked to the lack of one or more genes in the nitrate assimilation gene cluster, containing a nitrate transporter, nitrate reductase, nitrite reductase and two Zn(II)2Cys6 type transcription factors . Despite the clear advantages of the ability to utilize nitrate in certain niches, the cost-benefit balance of nitrate utilization may favor the loss of this feature in certain niches and explain the observed diversity of this trait between different strains [76, 77, 84]. However, to support this hypothesis, more research is needed using a broad collection of ecologically and geographically diverse strains. Further, gene content analysis revealed a relative enrichment in cell-membrane related genes compared to closely related species and *S. cerevisiae* [61]. While not yet proven for *Brettanomyces*, these genes could be advantageous for survival in wine or beer stored in oak barrels, where these genes may mediate the adhesion of cells to the barrels and protect them from washing out during cleaning of the casks [86]. Furthermore, some *Brettanomyces* strains have the ability to hydrolyze cellobiose through β-glucosidase activity, and further ferment it to ethanol [87]. Cellobiose is a disaccharide obtained by hydrolysis of cellulose, a complex sugar present in, for example, wood, and may be obtained by the yeast from the internal wall of the wooden barrels, and thus help explain how *Brettanomyces* can survive for years in wooden casks. Finally, *Brettanomyces* is able to utilize and ferment a broad range of carbon sources, which is, however, variable between strains [77]. Additionally, different sugars seem to be fermented at different rates. For example, *B. bruxellensis* is able to ferment maltose and fructose, but at a lower rate compared to glucose [80]. Moreover, *B. bruxellensis* shows

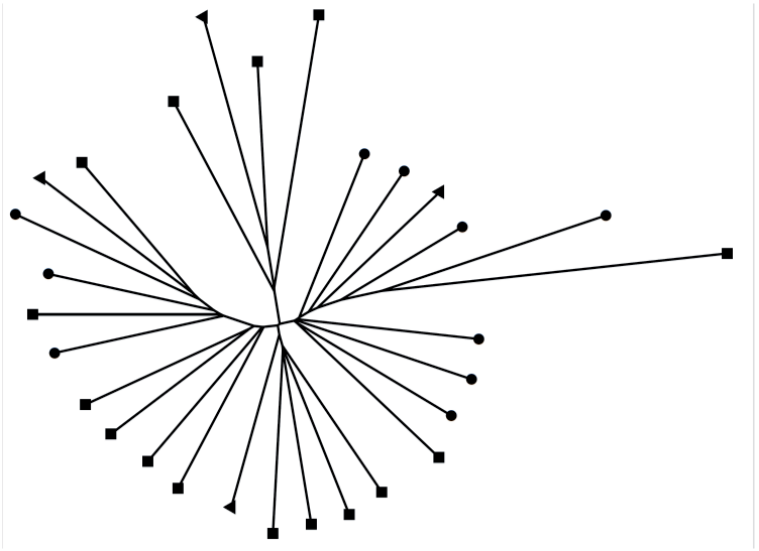

Fig. 2 **Neighbourhood-joining tree showing phylogenetic relations between *Brettanomyces bruxellensis* strains isolated from different sources, including beer (square), soft drink (triangle), and wine (circle). Studied strains were genotyped using seven established DNA fingerprinting techniques. Data analysis was performed on the combined dataset as described in Crauwels et al. (2014) [76]. Results suggest a correlation between *B. bruxellensis* genotypes and source of isolation**

very efficient sucrose utilization which may be the key for the high competiveness of *B. bruxellensis* in sucrose-based fermentations [85, 88]. Further, *B. bruxellensis* shows a higher affinity for glucose in carbon-limiting conditions. More importantly, *Brettanomyces* is also able to degrade and ferment more complex sugars that are not fermentable by *Saccharomyces* such as cellobiose (see above) and dextrins. Dextrins such as maltotetraose and maltopentaose are often present as residual sugars after the main alcoholic fermentation by *Saccharomyces*. Using its α-glucosidase activity *Brettanomyces* is able to hydrolyze these sugars [89], yielding superattenuated beers with slightly higher ethanol levels and lower caloric contents.

6 Differences between wine spoilage and beer brewing *B. bruxellensis* strains?

Genetic diversity studies have revealed significant genotypic variability within the species *B. bruxellensis* [76, 90-93]. Moreover, some of these studies report a correlation between genotype groups of *B. bruxellensis* and their source of isolation (e.g. beer or wine) (Fig. 2) [76, 81, 93], suggesting niche adaptation. Furthermore, recently, this correlation was also suggested for *B. bruxellensis* phenotypes although only a limited set of seven isolates was studied (2 wine strains, 4 beer strains and 1 from soft drink) [77]. The ability to metabolize particular α- and β-glycosides as well as α- and β-substituted monosaccharides was found to be highly variable between *B. bruxellensis* strains, but consistent for strains from the same origin. While strains isolated from wine were able to utilize D-galactose, this seems less the case for beer isolates. Accordingly, strains unable to grow on galactose were found to lack at least one of the genes involved in the Leloir pathway of the galactose metabolism. Further, in contrast to wine strains, brewing strains were found to be not capable of hydrolyzing the β-glycoside disaccharides cellobiose and gentiobiose, suggesting that these strains lack the enzyme(s) responsible for the breakage of specific β-bounded sugars. Indeed, whole genome sequencing revealed that while the studied wine strains contain two (distinct) β-glucosidase genes, the investigated beer strains lack one of these genes [77], which may explain these phenotypic differences. However, according to Verachtert and De Mot [94], 43.3 % of 147 tested *Brettanomyces* strains from lambic fermentations (identified as *B. custersii* and *B. intermedius* at that time, two species that have now been reclassified as *B. bruxellensis*) were found to possess cellobiase activity, suggesting that *B. bruxellensis* brewing strains also harbor cellobiose-positive phenotypes. Further research using more isolates from different ecological niches is needed to investigate to what extent these finding represents a general trend for *B. bruxellensis* beer and wine strains.

Interestingly, the genes involved in the Leloir pathway as well as the above mentioned β-glucosidase gene absent in the beer strains are clustered in a ~36 kb region encompassing 13 genes, the majority of which are involved in carbon metabolism. This region was found to be completely absent in the beer strain ST05/12.22 [76]. Moreover, a more thorough study using PCR revealed that this gene cluster has been gradually lost over time in beer strains: some lack only a few genes, other lack all 13 genes, but all beer strains lack the β-glucosidase gene. In contrast, this cluster of genes was entirely present in wine strains (at least in eight of the nine studied strains) [77]. Furthermore, this region is also prone to copy number variations and loss-of-heterozygosity [77]. Based on these findings it may be speculated that this gene cluster carries a fitness cost for *B. bruxellensis* in certain fermentation systems such as beer brewing, thereby providing a selective pressure for its loss.

It is also interesting to note the possibility that the yeast ploidy level may be linked to its ecological niche. More particularly, triploidy seems to be predominant in the Australian *B. bruxellensis* population, since it is observed in 92 % of all isolates from Australian wines [84, 95]. Moreover, microsatellite typing suggests the existence of similar populations in French and South-African wineries [96]. In contrast, the majority of *B. bruxellensis* beer strains investigated to date are found to be diploid [77]. Triploid strains contain a core diploid genome (comparable with diploid strains) and a third distinct haploid complement, which they may have obtained through interspecific hybridization [84]. This intriguing genome structure is not rare in yeasts and resembles the interspecific hybrids identified in the *Saccharomyces* sensu stricto clade, such as the lager yeast *Saccharomyces* pastorianus and the *S. cerevisiae/S. kudriavzevii* hybrids isolated from wine and ale beer fermentations [97, 98]. In the case of *Saccharomyces* hybrids, it was hypothesized that the additional set of chromosomes confers a selective advantage in an industrial environment, but it remains to be determined whether a similar scenario is at play in *B. bruxellensis*. It was suggested that the ability of most wine strains to withstand high levels of sulfite, the main anti-spoilage agent in wine fermentations, might be (at least partially) explained by the triploidy state [61, 84]. However, although these preliminary investigations hint towards certain trends between the genetic structure as well as the phenotypic behavior of *B. bruxellensis* populations and their source of origin, more elaborate studies using large collections of diverse strains, are needed to draw strong conclusions in this regard. Such study may also reveal unintended human influences on the evolution of *B. bruxellensis*, e.g. with regard to traits such as tolerance to sulfite.

7 Methods of detection and identification

Due to *Brettanomyces*' economic importance as spoilage yeast much attention has been given in the past to the development of reliable detection methods. Advantageously, the same detection tools can be used to monitor or characterize beneficial brewing strains. For example, semi-selective media with ethanol as carbon source and phenolic precursors such as hydroxycinnamic acids have been used to favor the growth of *Brettanomyces* over other yeasts [99]. Subsequent identification to the species level can be performed by several methods, including sequencing of (part of the) ribosomal RNA (rRNA) genes. A major drawback of these culture-based methods, however, is the long incubation period needed to cultivate the yeast under laboratory conditions. Much faster are molecular methods, enabling specific and sensitive detection of the yeast without cultivation [100]. Available molecular detection methods for *Brettanomyces* include, for example, a nested PCR targeting a specific *Brettanomyces* DNA fragment [101101] and a PCR-restriction enzyme analysis protocol based on part of the large subunit of the rRNA gene [102]. Additionally, species-specific PCR assays have been developed based on polymorphisms in the

internal transcribed spacer (ITS) region of the rRNA genes [35]. Further, a quantitative real-time PCR has been developed enabling simultaneous detection and enumeration (quantification) of *B. bruxellensis* in wine [103]. However, whereas these PCR-based assays generally perform well, problems related to PCR inhibitors that are co-extracted during DNA extraction may arise. In addition, DNA-based detection assays make no distinction between living and dead propagules, which may complicate interpretation of the results [104]. Further, DNA fingerprinting methods such as PCR restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), arbitrarily primed PCR (AP-PCR) and microsatellite fingerprinting [35, 49, 76, 90-92, 96, 105, 106] have been developed by which individual isolates can be characterized and discriminated. Using these techniques, different *Brettanomyces* isolates can be efficiently compared based on their genetic content, e.g. enabling lambic brewers to characterize their *Brettanomyces* population to the strain level.

8 Opportunities for the brewing industry

It is clear from above that *Brettanomyces*, and especially *B. bruxellensis* as the most investigated species so far, holds an incredible potential to be exploited on a larger scale by the brewing industry. The unique aromatic properties and flavoring capability of the species are increasingly recognized in the food and beverage industry as well as by the consumer worldwide. Indeed, lambic and gueuze beers are increasingly attracting interest outside Belgium (where they originate from), especially in the USA where craft-brewers try to mimic the lambic beer production method to produce American coolship ales [13]. Furthermore, because of the growing interest in beers of spontaneous fermentation, some traditional breweries start making industrially produced lambic beers, by which a higher production capacity can be reached [107]. Industrial lambic breweries generally filter, pasteurize and carbonate their spontaneously fermented beers, which are sometimes also sweetened [41]. Moreover, they can brew lambic-type beers continuously as they generally have the capacity to prechill the wort before it is transported into the coolship, and hence do not need cold winter months to properly cool their wort in one night. Additionally, industrial brewers generally use larger wooden casks for beer maturation instead of the smaller wine or cognac casks that are commonly used by traditional brewers. Interestingly, comparison of the microbial diversity of industrially produced lambic beer with that of traditionally produced lambic beer revealed that both fermentations shared a core microbiota, consisting of *S. cerevisiae, S. pastorianus, B. bruxellensis* and *Pediococcus damnosus* responsible for the main fermentation and maturation phases [107]. It was hypothesized by the authors that these microbes originated from the wood of the casks, emphasizing the important role of the wooden casks used for the fermentation. This also suggests that lambic-like beer styles can be produced in a more industrial way, without losing the typical microbial successions seen in traditionally brewed lambic beers. Furthermore, more and more (artisan) breweries are intentionally adding *Brettanomyces* to their fermentations, either as a pure culture or in combination with more traditional brewing strains, aiming to develop novel alcoholic beverages. Additionally, an increasing number of brewers have a strong interest in maturing existing beer styles in wooden casks to provide sourness and additional aromatic notes to the beers, sometimes accompanied with a change in their color (cfr. Bersalis Triple Oak Aged, Oud Beersel, Belgium) [108]. In short, it can be expected that several novel alcoholic beverages based on (re-)fermentation with *Brettanomyces* will be introduced in the market in the near future. However, as previous studies have shown considerable genetic and phenotypic variation within *B. bruxellensis*, it may be expected that these differences also translate into a distinct impact on flavor development in beer fermentations. Production of the typical Brett volatile compounds is affected by various environmental conditions (ethanol content, pH, sugar and oxygen concentration,…) and the availability and composition of the necessary precursors (hydroxycinnamic acids) [64, 109]. Further, there is growing evidence that also the yeast strain has an important role in flavor production, with one strain more efficient than another [63, 64, 110; *Crauwels* et al., manuscript in preparation]. This also implies that, whenever adding a starter culture, a well-thought choice of the isolate(s) to be used should be based on scientific, rather than on e.g. historical grounds for successful fermentation. Moreover, strain selection should also consider potential health issues, since it is reported that some *Brettanomyces* strains can produce biogenic amines [20], which are hazardous biological compounds that can have undesirable physiological effects when absorbed in high concentrations. To this end, large collections of different strains should be screened for relevant parameters, enabling the selection of a superior strain resulting in high-quality beers.

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